HISTOPHYSIOLOGY OF GILL AND KIDNEY OF CRAB OCYPODE ALBICANS

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Brachyuran crabs possess the ability to regulate the internal level of the chloride ion against shifts in the external level of this ion. Webb (1940) and Jones (1941) showed that this ability varies in different species of crabs, and becomes functional over an increasingly wider range as the animals move away from the sea into an estuarine or shore habitat. Data presented by Flemister and Flemister (1951) indicated that the ghost crab, *Ocypode albicans* (Bosq), is able to regulate the internal chloride ion against a hypotonic environment of 200 millimoles of chloride per liter, and a hypertonic environment of 600 millimoles per liter. That is, within this range of environmental chloride ion, the internal chloride ion of the crab is maintained at 375 millimoles per liter. Such regulation is accomplished by reciprocal mechanisms for uptake and loss or absorption and secretion of the chloride ion, these mechanisms being located in cells which occupy appropriate sites in reference to the external and internal environments.

The principal site of chloride ion uptake by decapod Crustacea is thought to be the gills. The uptake of ions from the environment by fresh water animals was reported by Krogh (1937) as a probable function of the gill. Webb (1940) suggested that the histology of the gill of *Carcinus maenas* was compatible with the process of salt and water transfer. Isolated gills of the crab *Eriocheir sinensis* were shown to absorb ions from the environment by Koch, Evans and Schicks (1954). Chloride ions are excreted from the body by the kidney or antennal gland. It has been established that under conditions of excess chloride ion in the environment, the urine secreted by the kidney contains a higher proportion of chloride ion than it does under conditions of low chloride ion in the environment. An investigation of the relation of oxygen consumption to chloride ion regulation reported by Flemister and Flemister (1951) led to the conclusion that chloride ion regulation by the kidney was supplemented by the activity of some other tissue or tissues. Excretory cells are found in the gill and in the hepato-pancreas, in addition to the kidney. Early accounts of crustacean anatomy by Cuénot (1895) and Pearson (1908) describe these cells and attribute an excretory function to them. A more recent study by Lison (1942) emphasizes their possible excretory role. These findings suggest that gill tissue may be active in chloride ion loss, as well as uptake. The hepato-pancreas is likewise a site at which chloride ion may be absorbed or excreted, although its role in regulation is doubtful. Travis (1955) has described the functional histology of this structure in detail. Similarly, the tufts of branchial epithelium

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which partially line the gill chamber of *Ocypode albicans* occupy a position in which they might contribute to the transfer of ions and water. An investigation of the histophysiology of gill, kidney and branchial epithelium of *Ocypode albicans* was undertaken with the special objective of examining their role in the transfer of the chloride ion.

Methods

Ocypode albicans was collected in the summer months on the beaches at Rehoboth, Delaware, and at Bermuda in March and April. The animals were brought into the laboratory where they were maintained on damp sand and offered food until they were subjected to experimental treatment. The entire period of their stay in the laboratory was not longer than ten days or two weeks. No animals showed indications of impending molting and none molted in the laboratory.

Animals were acclimatized in sea water containing, respectively, 200, 400 and 600 millimoles of chloride per liter. The chloride ion level of the blood of *Ocypode albicans* is maintained in the range of 375 to 400 millimoles of chloride per liter; thus the range of salinities was hypotonic, isotonic and hypertonic in reference to the internal chloride ion concentration. Animals remained in the experimental tanks for seventy-two hours. Blood and urine were collected and analyzed for chloride ion content before and after acclimatization, using methods described in a previous paper (Flemister and Flemister, 1951).

At the end of the period of acclimatization, tissues were removed for study. Tissues taken included gill and antennal gland, which were fixed without further dissection, and the branchial epithelium. This latter tissue in *Ocypode albicans* is the tufted lining of the inner face of the branchial chamber. It was removed, cut into two portions and these fixed flattened out. Fixation in formalin or Bouin's fixative, followed by hematoxylin and eosin or Mallory's connective tissue stain, was used for general histological examination. Regaud's fixative followed by post-chroming resulted in fixation of mitochondria which were then stained by iron hematoxylin or Altman's acid aniline fuchsin.

The Leschke method for the detection of chlorides was used according to the sequence described by Copeland (1948). Tissues were removed from the animal and fixed, without washing, in one per cent silver nitrate made acid with nitric acid. This fixation was accomplished in the dark, as was the development in Eastman D-11 (diluted 1:4) and final fixing in Eastman F-5 (diluted 1:5). The tissues were then washed, dehydrated, embedded in paraffin and sectioned. Sections were gold toned and sometimes counter-stained with eosin. The picture of silver deposition which resulted, as will be discussed below, led to testing for a clue as to the nature of the material reacting with the silver. Polyphenols, urates and fats might possibly be expected to react with the silver in the procedure described. These were individually tested for by the following procedures.

The Hollande method for the detection of urates was used as described by Glick (1948). This involved fixation in one per cent silver nitrate solution in neutral formalin, in the dark. Polyphenols were tested for by treating sections of formalin-fixed material in ammoniacal silver nitrate in the dark by Masson's method (Lison, 1936). Reduced silver deposits in each of these indicates presence of material tested for. Some formalin-fixed tissues were washed and imbedded in Carbowax

after the method of Blank and McCarthy (1950) and the sections stained with Sudan III for the detection of fats.

Results

The gill

Descriptions of the histology of decapod crustacean gills are to be found in the paper of Cuénot (1895) and the monograph on *Cancer* by Pearson (1908). The gill of *Ocypode albicans*, as that of other brachyurans, is formed of a number of lamellae, or broad flattened plates arranged serially in pairs along a central gill stem (Fig. 1). The gill stem provides support for the lamellae and is the pathway for the afferent and efferent branchial vessels. The entire outer surface of the gill is covered by a thin layer of chitin which is about 1 μ in thickness.

The individual leaflet or lamella may be likened to a flattened thin-walled sac. Underlying the chitin is a continuous lining of epithelial cells (Fig. 2). At irregular intervals the faces of the lamella are joined by large cells whose cytoplasm contains distinct fibrils, and which constitute pillar cells. The distal border of the lamella is expanded, being free of pillar cells and traversed by an occasional connective tissue fiber. The irregular cavity within the lamella resulting from this arrangement of pillar cells and fibers is filled with blood in life and an occasional blood cell is seen in sections. The lamellar blood space communicates with the afferent and efferent branchial vessels. The epithelial cells of the lamellae are continued as the lining of the gill stem. Collagen fibers of some thickness are found in the stem itself. Large connective tissue cells conforming to the classification of Leydig cells of the first order, according to Kükenthal (1926–1927), compose the chief support of the gill stem. Smaller spindle-shaped Leydig cells of the third order may also be found. Blood cells are commonly seen lying in the interstitial spaces.

In addition to the cells described, there is yet another type which appears to be unique to the gill. It was termed a branchial excretory cell by Cuénot (1895) and a branchial athrocyte by Lison (1942). These cells are large, oval in shape, with the cytoplasm arranged in a peripheral layer surrounding a vacuole. The nucleus is displaced to one side, close to the cell membrane. The vacuole contains material which in fixed sections appears as an aggregate of granules incompletely filling the space. The cells are arranged in irregular rows or aggregates which protrude into and are bathed by the blood which passes through the stem. Lison (1942) and earlier investigators have observed that certain classes of dyes injected into the blood stream may be accumulated by these cells and from this function is derived their classification as branchial excretory cells.

In mitochondrial preparations it is immediately apparent that the lamellar epithelium is rich in mitochondria, while the branchial excretory cells show a sparse or absent population (Fig. 3). It is also apparent that although the lamellar epithelium is continuous with the lining of the gill stem, there is a marked decrease in the mitochondrial count in the cells lining the gill stem. The mitochondria may be filamentous or arranged in minute rows of granules; they occupy the cytoplasm of the cell on either side of the nucleus, and do not appear to be constantly located either toward the base or toward the distal surface (Fig. 4). In preparations made

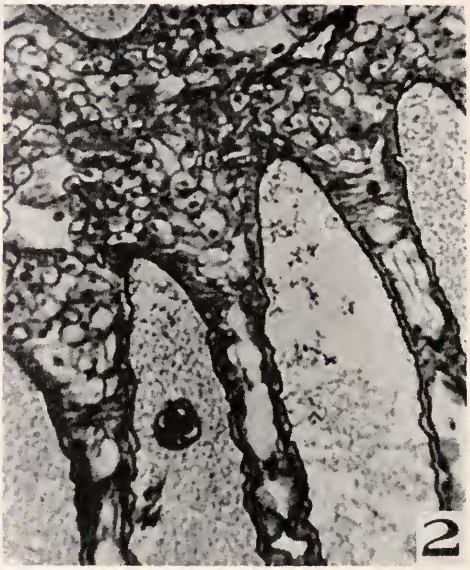


FIGURE 1. Portion of gill. 17×. Several lamellae attached to central stem. Afferent, ventral and efferent, dorsal, vessels appear as light, roughly circular areas. Darker part of stem is area of branchial athrocytes.

FIGURE 2. Longitudinal section of stem of gill with several lamellae. 200×. Note loosely packed athrocytes, blood spaces and occasional connective tissue fibers in stem. Lamellae are lined by epithelium below a thin chitin cover. Pillar cells appear to join faces of lamellae.

FIGURE 3. Portion of stem and lamella stained with iron hematoxylin. 380×. Mitochondria show as dark clumps in lamellar epithelium. Athrocyte in extreme left center field shows enclosed material lightly stained. Very dark cells in stem are blood cells.

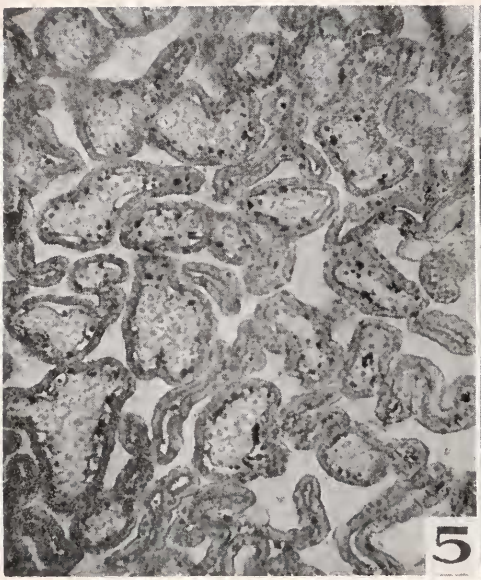
FIGURE 4. Section of lamella, iron hematoxylin. 860×. Mitochondria appear as dark clumps and threads. Chitin covering heavily stained.

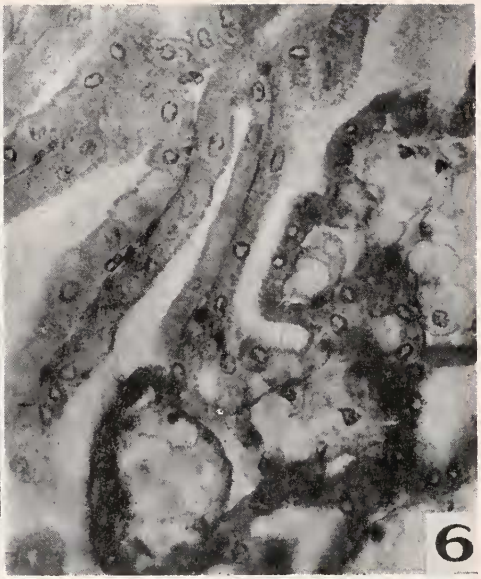
from crabs acclimatized to 200 millimoles of chloride per liter the lamellar cells show some vacuolation; in such instances the mitochondria line up at the borders of the vacuoles. Vacuolation is less in lamellar cells of material taken from crabs which had been living in sea water containing 400 and 600 millimoles of chloride per liter, but otherwise the picture is the same. Mitochondria appear as scattered granules in the peripheral cytoplasm of the branchial excretory cells. However, the material present in the vacuoles of these cells often stained with the mitochondrial stain.

The Leschke test for the detection of chlorides gave clear results on one point: there is never, under any circumstances, any evidence of silver deposition in the lamellar epithelium, the epithelium of the stem, any of the connective tissue of the stem, or in any of the blood cells. There is blackening of the covering chitin, as would be expected since the tissues were not washed before fixing. There is occasional outlining of the nucleus, and connective tissue fibers may show blackening. In all of the tissues prepared from Rehoboth Beach crabs there is blackening of the material contained within the vacuole of the branchial excretory cells. This is not uniformly true of the crabs taken in Bermuda. Careful examination of material taken from crabs from the three classes of environmental situations shows no obvious quantitative differences which might reflect activity in the regulatory mechanism.

The interpretation of the occurrence of deposits of reduced silver as evidence of the localization of chloride is open to dispute. The Leschke method depends upon the formation of silver chloride after treatment with acid silver nitrate and subsequent reduction of silver by means of a quinone-containing solution. Such a method was meticulously explored by MacCallum (1905) using a variety of biological materials. It was his conclusion that only halides of all substances in biological materials would give this particular reaction. It is to be noted that in his experiments, proteins and other substances used were purified by repeated treatment to rid them of all free chloride. His conclusions have been used by a number of workers to justify their results with the Leschke method. Keys and Willmer (1932) used this method in determining the location of the "salt cells" of fish.

There are two lines of objection to a blanket acceptance of silver deposits as evidence of the localization of chlorides. One of these is based on the highly diffusible nature of the chloride ion which tends to move freely in biological fluids and across membranes. Silver is a heavy metal and tends to be adsorbed upon membranes so that penetration of the solution may be uneven. Thus, any reduced silver found after the treatment is complete may, or may not, represent a true localization of chloride present *in vivo*. The second objection, which in some respects is more serious, is that certain substances found in tissues are capable of immediately reducing acid silver nitrate in the dark. The best known of these is Vitamin C, ascorbic acid, and this property is the basis of the method of Giroud (1938) for determining the location of Vitamin C in tissues. Polyphenols give a silver precipitate after treatment with ammoniacal silver nitrate, and urates yield a silver precipitate after treatment in neutral silver nitrate. Tissues from *Ocypode albicans* subjected to these tests showed no evidence of reduced silver in the locations observed after the Leschke test or in any other locations. Vitamin C is regularly found in the cytoplasm of the cell, occupying a position in the neighborhood of the Golgi apparatus. Thus the nature of the material contained within the vacuoles of the branchial





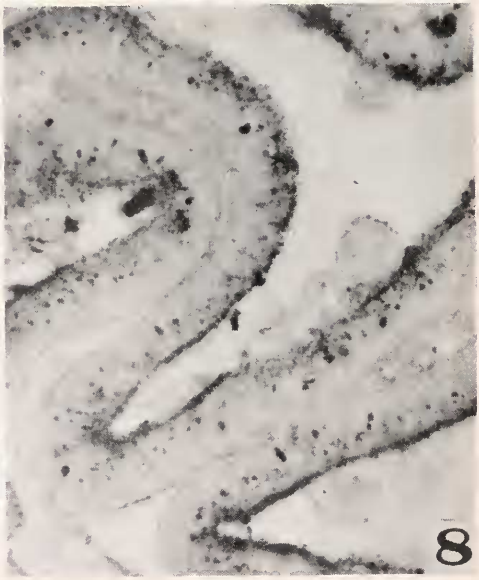


FIGURE 5. Section of kidney, hematoxylin and eosin-stained. 100 ×. Typical view of labyrinth, showing renal tubule cells surrounding saccule cells. The latter are very pale. Occasional very dark cells between the two layers are blood cells in the blood spaces.

FIGURE 6. Labyrinth, hematoxylin and eosin. 380 ×. The lower left hand portion of the picture shows renal tubule cells. Note brush border, and the secretion bleb being extruded into the lumen. In the upper right hand corner are a few saccule cells, very pale and appearing almost empty.

FIGURE 7. Labyrinth, iron hematoxylin. 380 ×. At top renal tubule cells surround saccule cells which have not been stained by iron hematoxylin. Section of renal tubule at bottom shows mitochondria situated towards the hemocoele, in the basal portion of the cells.

excretory cells is still undetermined. It seems reasonable to speculate, however, that under some circumstances this might be a complex molecule which is capable of combining loosely with chloride. An alternate interpretation would be that it is a molecule which under some circumstances carries a reducing radical of yet undetermined nature. The material is not fat; it persists after normal dehydration and clearing procedures; it stains with cytoplasmic stains generally; it does not stain with Sudan III after Carbowax embedding and sectioning. What it is remains undetermined; that the reduced silver indicates the location of a chloride remains to be positively substantiated.

The kidney

The histology of decapod crustacean kidneys is amply treated in the descriptive works of Marchal (1892) and Pearson (1908). The kidney of *Ocypode albicans* conforms to these descriptions. The labyrinthine structure is the result of the growth in close proximity of two sac-like portions of the excretory tubule. The floor of the more dorsal end sac pushes into the roof of the more ventral renal tubule with a consequent close interdigitation of the layers. The lumen of the end sac communicates with the lumen of the renal tubule which in turn empties through a bladder to the outside. There is no direct connection between the hemocoele and the lumen of the excretory apparatus; all materials eliminated must pass through the cells either of the end sac or renal tubule.

Typical sections through the kidney show portions of the end sac, renal tubule, and areas of interdigitation (Fig. 5). Comparisons of end sac epithelium and renal tubule epithelium can be made easily in the areas of interdigitation, at which locations the end sac epithelium always constitutes the inner layer of cells, surrounded by an outer layer of renal tubule epithelium. The appearance and staining capacities are sufficiently different so that renal tubule cells may always be distinguished from end sac cells. Renal tubule cells are cuboidal in shape, stain deeply and have a well defined brush border on the surface of the cell facing the lumen (Fig. 6). In contrast the cells of the end sac are large oval or cuboidal cells generally arranged in one layer although they may occasionally form two indistinct layers. A considerable portion of the cell is occupied by a vacuole which may contain granular material, and the nucleus is consequently displaced to one side. Staining is invariably light or pale in contrast to the deeper staining renal tubule cells. The end sac cells resemble closely the branchial excretory cells described for the gill, and they have been termed kidney athrocytes by Lison (1942).

Mitochondria are found in renal tubule cells, usually as filaments occupying the area of the cell towards the hemocoele (Fig. 7). There are generally few or no mitochondria observed in the end sac cells, and the material of the vacuole does not stain with the mitochondrial stain.

Kidney tissue treated by the Leschke method shows silver deposition as follows. The contents of the vacuoles of the end sac cells are blackened. The degree of blackening is not uniform, and shows no correlation with the observed regulatory

FIGURE 8. Labyrinth, silver fixation. 860 ×. Portion of renal tubule from crab acclimatized to hypertonic environment. Heavy deposition of silver apparently in brush border area of cell, and outlining secretion blebs. Note that silver also accumulates within cell in discrete particles. They are not nuclei, and significance of their occurrence is not known.

activity of the animal. Tests similar to those made on gill tissues for polyphenols, urates and fats gave negative results. The nature of this material is undetermined. In kidney tissue removed from crabs acclimatized to 600 millimoles of chloride per liter there is a pronounced deposit on the lumen side of the renal tubule cell (Fig. 8). This blackening appears to involve the brush border as well as the immediately adjacent lumen edge of the cell. The blebs of secretion characteristic of this type of cell are also outlined with faint depositions of silver. The cytological picture here coincides with the known physiological activity of the kidney, which is excretion of excess chloride under the conditions of regulation to the hypertonic environment. There seems to be little doubt that the site of excretion is through the renal tubule cells. Silver deposition in kidneys taken from animals acclimatized to 200 millimoles of chloride per liter shows along the lumen border of cells, but not outlining the secretion blebs; the silver always seems to be within the cell. In several specimens from Bermuda, the sections show no silver except at the blood side of the cell. This was not observed uniformly in the tissues of crabs acclimatized to a hypotonic environment. Under such conditions the kidney is excreting a dilute urine with reference to the chloride content and it is perhaps impossible to detect accumulation or reabsorption of chloride which might be occurring.

The branchial epithelium

The lining of the inner surface of the gill chamber is the branchial epithelium, which in *Ocypode albicans* is developed into numerous tufts which presumably offer increased surface for respiratory exchange. The membrane is composed chiefly of a large blood space surrounded by the stellate and spindle-shaped connective tissue cells characteristic of Crustacea. The main blood channel protrudes into finger-like projections which follow poorly defined ridges. Within the network of connective tissue are scattered large cells corresponding to the reserve cells of Cuénot (1895). These cells have the property of accumulation and storage of proteinaceous materials. The outer covering is composed of a single layer of flattened epithelium covered by a very thin layer of chitin.

None of the cell types show any evidence of possible active absorption or secretion. The mitochondrial picture does not indicate such activity on the part of any cells; there are no athrocytes; and there is no evidence of tegmental glands associated with this epithelium. It seems probable that respiratory exchange occurs across this membrane, although the contiguity of external and internal environments is not as close as in the gill lamellae. Although salt transfer, and that of water, could be accomplished by direct osmotic forces it does not seem possible that this membrane takes an active part in salt and water regulation.

Discussion

The microscopic anatomy of the gill and kidney of *Ocypode albicans* can be related to the function of transfer of chloride ion between the blood of the animal and the environment. The hepato-pancreas and the branchial epithelium are likewise areas at which transfer may occur, but these sites do not appear to be concerned with regulation. Gill and kidney on the other hand are not only areas of passive transfer, but are also elements of the regulatory mechanism. The regulatory mechanism has

been demonstrated by Flemister and Flemister (1951) to be effective in *Ocypode albicans* over a hypotonic and hypertonic range of environmental chloride ion content, and it has been further demonstrated that this regulation requires the expenditure of energy.

The chief portal of entry of the chloride ion into the body of the crab is at the gill surface. The lamellar cells which constitute the cellular surface of the gill are closely associated with the blood stream, which bathes one surface, and the external environment which bathes the other surface. The cells give evidence of being active in some secretion or absorption process by their rich population of mitochondria, which is a generally accepted sign of a metabolically active cell. The lamellar cells under no circumstances showed any evidence of accumulation of chloride ion, and it is assumed that the absorption process is a continuing one and does not involve even temporary accumulation within the cell. These same cells are the final route of

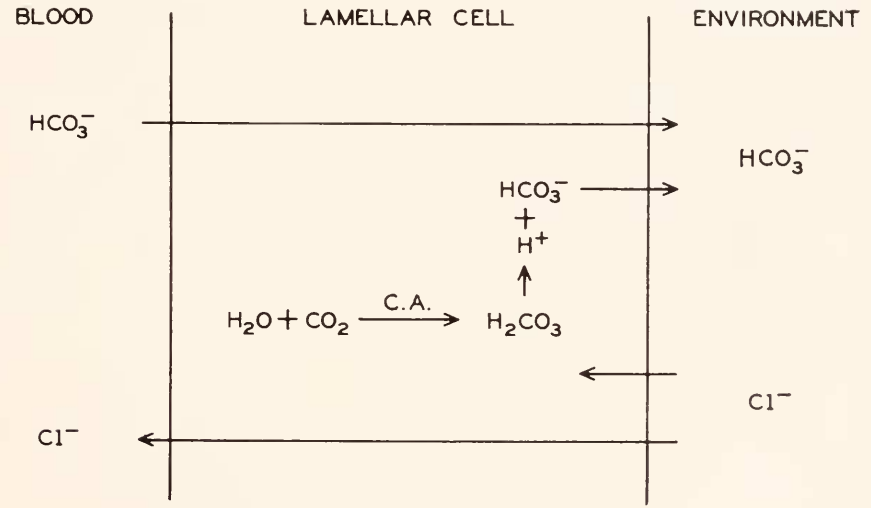


FIGURE 9.

carbon dioxide as it leaves the body. It is known from the work of Ferguson, Lewis and Smith (1937) that the gills of crustaceans contain a large amount of the enzyme carbonic anhydrase as compared with the blood or other tissues taken from the animals. The diagram (Fig. 9) shows how the excretion of carbon dioxide may be related to the function of chloride ion uptake by the gill of the crab. Carbon dioxide from the body tissues arrives at the lamellar cell in the form of bicarbonate, and is transferred across the cell to the environment. As bicarbonate leaves the cell, chloride ion enters, maintaining the ionic balance; similar exchange occurs at the blood surface of the cell, with the net result that chloride ion enters the blood stream as carbon dioxide is lost from the body. This uptake is augmented by some specific cellular activity which results in the production of carbon dioxide which is removed from the cell as bicarbonate produced by the activity of carbonic anhydrase. It is believed that this absorption mechanism is working under all conditions of tonicity of the environment, and is independent of the ion content of the environment. It

is effective in supplying enough chloride ion to maintain the internal level until the external level falls below about twenty-five per cent of the internal level (Flemister and Flemister, 1951). Below this level it is still working as can be observed from the uptake experiments cited above. When the crab is subjected to a hypertonic environment, the mechanism is still at work, and supplements the osmotic force tending to drive chloride ion into the body. The internal chloride ion level then is maintained by excretion of excess chloride ion by the kidney, and perhaps at other sites. It is interesting to note that in *Gecarcinus lateralis* as reported by Flemister (1958) the blood chloride ion level tends to rise above the normal level when the crab is living in hypertonic environment. It appears that the excretory mechanism cannot keep up with the intake in this particular situation.

The renal tubule cells of the kidney are the principal sites of chloride ion excretion. The cells lie between the blood stream and the lumen of the kidney which communicates with the exterior; they show the brush border and mitochondria characteristically associated with absorbing or secreting cells; it is reported by Krugler and Burkner (1948) that alkaline phosphatase is found in these cells; and the cytological picture resulting from the Leschke test adds evidence that chloride ion may be excreted from the body at this site. Analysis of the urine of crabs reported by Flemister and Flemister (1951) showed that there is always chloride ion in the urine, and the amount increases directly with increasing chloride ion in the environment. The mechanism of secretion is not known, but it appears to be a function of the renal tubule cells. There is no evidence here that these cells can reabsorb chloride ion from the urine. Hence the renal tubule cells are the route of chloride ion out of the body.

The athrocytes of the end-sac and the branchial athrocytes are concerned with the removal of large, poorly diffusible molecules from the blood stream. According to Lison (1942) this is accomplished by a process of accumulation, the exact nature of which is not understood. Final removal from the body results from the breaking away of the end-sac athrocytes so that they float freely in the lumen of the kidney and are lost from the body with the urine. There is no such obvious final route for the gill-stem athrocytes, and they may perhaps be looked upon as analogous to the fixed macrophages of the vertebrate reticulo-endothelial system. However, it should be noted that specific evidence for a phagocytic action is lacking. It is difficult to imagine how the athrocytes could be involved in the mechanism resulting in chloride ion regulation, unless the ion is somehow attached to a large poorly diffusible molecule within the cell, and thus removed from the blood stream. If the Leschke test is assumed valid as an indicator of the presence of halides, then the athrocytes contain halides in quantity far greater than other cells. There is no quantitative difference in the amount of halide bound by the athrocytes of crabs taken from hypotonic or hypertonic environments. Perhaps the only function of these cells is accumulation, and there can be no eventual release back into the blood stream. These cells would then act antagonistically to the lamellar cells which are continually absorbing chloride ion from the environment, and they would supplement the function of the renal tubule cells. It is to be noted that this speculation is based on an assumed validity of the Leschke test.

There is some indication that the kidney is not the only site of chloride ion loss from the body, according to Flemister (1958). Granting that the role of the

athrocytes is hypothetical, one other source of leaking of chloride ion could be at the branchial epithelial surface. Since this surface is relatively thin and lies between blood stream and environment, there is ample opportunity here for exchange of ions as a result of osmotic differentials. Thus the branchial epithelium might serve as a portal of entry of chloride ion in a hypertonic medium, a site of chloride ion loss in a hypotonic medium. The regulation of the blood chloride ion level must depend on those cells which are active in absorbing or secreting chloride ions, those of the lamella of the gill and the renal tubule of the kidney.

Summary

1. The fine structure of the gill and kidney of *Ocypode albicans* was examined for evidence of participation in the transfer of water and the chloride ion.

2. The epithelium of the gill lamellae was found to have the characteristics of a secreting epithelium. No other cells associated with the gill structure had either the position or morphology to be considered important in this function. The athrocytes of the gill stem probably do not participate in salt-water regulation, but are concerned principally with the removal of poorly diffusible ions from the blood stream. No mechanism of such removal can be interpreted from the present study.

3. The cells of the renal tubule of the kidney were found to have the characteristic brush border and mitochondrial picture associated with actively secreting or reabsorbing epithelia. Silver deposition following the Leschke test gave evidence that these cells are involved in the excretion of chloride, and may possibly also act to reabsorb chloride from the urine. The athrocytes of the kidney end-sac, like those of the gill stem, probably do not function in salt or water regulation. Materials accumulated within these cells are lost to the body when the cells break away and float free in the urine.

4. Absorption of salt from the environmental medium is accomplished by an energy-using mechanism in the lamellar cells. This is a constant function and is associated with the carbonic anhydrase mechanism working in the excretion of carbon dioxide. Excretion of salt by the renal tubule is probably also a constant function, although no evidence as to its possible mechanism is available. Reabsorption of salt at this location is a possibility; the triggering mechanism is probably a falling chloride concentration in the blood.

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